PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 39/39, 39/29, 39/145
A61K 9/00

A1

(11) International Publication Number: **WO 94/01133**

(43) International Publication Date: 20 January 1994 (20.01.94)

(21) International Application Number: PCT/US93/06298

(22) International Filing Date: 7 July 1993 (07.07.93)

(30) Priority data:
07/910,399 8 July 1992 (08.07.92) US

(60) Parent Application or Grant
(63) Related by Continuation
US 07/910,399 (CIP)
Filed on 8 July 1992 (08.07.92)

(71) Applicant *(for all designated States except US)*: SCHERING CORPORATION [US/US]; 2000 Galloping Hill Road, Kenilworth, NJ 07033 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : BONNEM, Eric, M. [US/US]; 20 Remington Road, Mt. Vernon, NH 03057 (US). CHAUDRY, Imtiaz, A. [US/US]; 18 Rose Avenue, North Caldwell, NJ 07006 (US). STUPAK, Elliot [US/US]; 11 Woodland Road, West Caldwell, NJ 07006 (US).

(74) Agents: LUNN, Paul, G. et al.; Schering-Plough Corporation, One Giralda Farms, M3W, Madison, NJ 07940-1000 (US).

(81) Designated States: AU, BB, BG, BR, BY, CA, CZ, FI, HU, JP, KR, KZ, LK, MG, MN, MW, NO, NZ, PL, PT, RO, RU, SD, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: USE OF GM-CSF AS A VACCINE ADJUVANT

(57) Abstract

The present invention is a method for enhancing the immune response of a mammal to a vaccine comprising administering to such a mammal an effective amount of GM-CSF in conjunction with a vaccine. The present invention further provides for a pharmaceutical composition comprising an effective amount of GM-CSF, a natural, synthetic or recombinant antigen and a pharmaceutically acceptable carrier.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**MR**	Mauritania
AU	Australia	**GA**	Gabon	**MW**	Malawi
BB	Barbados	**GB**	United Kingdom	**NE**	Niger
BE	Belgium	**GN**	Guinea	**NL**	Netherlands
BF	Burkina Faso	**GR**	Greece	**NO**	Norway
BG	Bulgaria	**HU**	Hungary	**NZ**	New Zealand
BJ	Benin	**IE**	Ireland	**PL**	Poland
BR	Brazil	**IT**	Italy	**PT**	Portugal
BY	Belarus	**JP**	Japan	**RO**	Romania
CA	Canada	**KP**	Democratic People's Republic of Korea	**RU**	Russian Federation
CF	Central African Republic			**SD**	Sudan
CG	Congo	**KR**	Republic of Korea	**SE**	Sweden
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovak Republic
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LU**	Luxembourg	**TD**	Chad
CS	Czechoslovakia	**LV**	Latvia	**TG**	Togo
CZ	Czech Republic	**MC**	Monaco	**UA**	Ukraine
DE	Germany	**MG**	Madagascar	**US**	United States of America
DK	Denmark	**ML**	Mali	**UZ**	Uzbekistan
ES	Spain	**MN**	Mongolia	**VN**	Viet Nam
FI	Finland				

USE OF GM-CSF AS A VACCINE ADJUVANT

FIELD OF THE INVENTION

The present invention relates to the use of granulocyte-macrophage-colony stimulating factor (GM-CSF), particularly human GM-CSF, as a vaccine adjuvant.

BACKGROUND OF THE INVENTION

Active immunization is the administration of an antigen to an animal to bring about an immune response in the animal. A vaccine against a microorganism is an antigenic preparation which when inoculated into a non-immune individual will confer active immunity to the microorganism but will not cause disease. Specificity and memory, the two key elements of the adaptive immune system, are exploited in vaccination, since the adaptive immune system mounts a much stronger response on second encounter with an antigen. This secondary immune response is both faster to appear and more effective than the primary response. The principle of vaccine development is to alter a microorganism or its toxins (natural antigens) in such a way that they become innocuous without losing antigenicity. Alternatively, antigenic polypeptides of the organism in question can be produced by recombinant methods or by synthetic chemistry to produce an effective vaccine.

One problem that frequently is encountered in the course of active immunization is that the antigens used in the vaccine are not sufficiently immunogenic to raise an antibody titer to sufficient levels to provide protection against subsequent challenge, or to maintain the potential for mounting these levels over extended time periods. Another problem is that the vaccine may be deficient in inducing cell-mediated immunity which is a primary immune defense against

bacterial and viral infection. Still another problem is that an individual patient might be immunosuppressed.

To obtain a stronger humoral and/or cellular response, it is common to administer a vaccine in a formulation containing an adjuvant. An adjuvant is a substance that enhances, nonspecifically, the immune response to an antigen, or which causes an individual to respond to an antigen who would otherwise without the adjuvant not respond to the antigen. An adjuvant is usually administered with an antigen, but may also be given before or after antigen administration. Suitable adjuvants for the vaccination of mammals include but are not limited to Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum monostearate); Freund's complete or incomplete adjuvant; mineral gels such as aluminum hydroxide, aluminum phosphate and alum; surfactants such as hexadecylamine, octadecylamine, lysolecithin, dimethyldioctadecyl-ammonium bromide, N,N-dioctadecyl-N',N'-bis(2-hydroxymethyl) propanediamine, methoxyhexadecylglycerol and pluronic polyols; polyanions such as pyran, dextran sulfate, poly IC, polyacrylic acid and carbopol; peptides such as muramyl dipeptide, dimethylglycine and tuftsin; and oil emulsions. The antigens could also be administered following incorporation into liposomes or other microcarriers.

SUMMARY OF THE INVENTION

It has been surprisingly discovered that granulocyte-macrophage-colony stimulating factor (GM-CSF) is an effective vaccine adjuvant.

Accordingly, the present invention provides a method for enhancing the immune response of a mammal to a vaccine

comprising administering to a mammal in need of vaccination an effective amount of GM-CSF in conjunction with a vaccine.

Preferably, the mammals treated will be humans and the GM-CSF utilized will be one of the human allotypes. Preferably, the GM-CSF will be administered from 1 to 14 days prior to or after the administration of the vaccine in an amount of about 0.1 to 100 micrograms (μg) per kilogram of body weight.

The present invention further provides for a pharmaceutical composition comprising an effective amount of GM-CSF, a natural, synthetic or recombinant antigen, and a pharmaceutically acceptable carrier.

Also claimed is a kit for enhancing an immunogenic response of a mammal to antigens in a vaccine comprising a first container having a pharmaceutical composition of GM-CSF contained therein; and a second container having a pharmaceutical composition of a vaccine contained therein.

DETAILED DESCRIPTION OF THE INVENTION

The teachings of the references cited in the present application are incorporated herein in their entirety by reference.

According to the present invention, we have surprisingly found that the immune response in a mammal, especially a human, to a vaccine can be effectively enhanced by the administration of an effective amount of GM-CSF in conjunction with the vaccine. The term "in conjunction with" as used herein refers to the administration of GM-CSF concurrently, before or following administration of a vaccine.

As used herein, "GM-CSF" means a protein which (a) has an amino acid sequence that is substantially identical to the sequence of mature (i.e., lacking a signal peptide) human GM-CSF described by Lee *et al. Proc. Natl. Acad. Sci. U.S.A.* 82 : 4360 (1985) and (b) has biological activity that is common to native GM-CSF.

Substantial identity of amino acid sequences means that the sequences are identical or differ by one or more amino acid alterations (deletions, additions, substitutions) that do not substantially impair biological activity. Among the human GM-CSFs, nucleotide sequence and amino acid heterogeneity have been observed. For example, both threonine and isoleucine have been observed at position 100 of human GM-CSF with respect to the N-terminal position of the amino acid sequence. Also, Schrimsher et al. [Biochem. J 247:195 (1987)] have disclosed a human GM-CSF variant in which the methionine residue at position 80 has been replaced by an isoleucine residue. GM-CSF of other species such as mice and gibbons (which contain only 3 methionines) and rats are also contemplated by this invention. Recombinant GM-CSFs produced in prokaryotic expression systems may also contain an additional N-terminal methionine residue, as is well known in the art. Any GM-CSF meeting the substantial identity requirement is included, whether glycosylated (i.e., from natural sources or from a eukaryotic expression system) or unglycosylated (i.e., from a prokaryotic expression system or chemical synthesis).

GM-CSF for use in this invention can be obtained from natural sources (U.S. Patent No. 4,438,032; Gasson et al., supra; Burgess et al., supra; Sparrow et al., supra; Wu et al., supra). GM-CSF having substantially the same amino acid sequence and the activity of naturally occurring GM-CSF may be employed in the present invention. Complementary DNAs (cDNAs) for GM-CSF have been cloned and sequenced by a number of laboratories, e.g. Gough *et al., Nature*, 309: 763 (1984) (mouse); Lee *et al., Proc. Natl. Acad. Sci. USA*, 82: 4360 (1985) (human); Wong *et al., Science*, 228: 810 (1985) (human

and gibbon); Cantrell *et al., Proc. Natl. Acad. Sci. USA*, 82 : 6250 (1985) (human), Gough *et al., Nature*, 309: 763 (1984) (mouse); Wong *et al., Science*, 228:810 (1985) (human and gibbon); Cantrell *et al., Proc. Natl. Acad. Sci. U.S.A.*, 82: 6250 (1985) (human).

GM-CSF can also be obtained from Immunex, Inc. of Seattle, Washington and Schering-Plough Corporation of Kenilworth, New Jersey and from Genzyme Corporation of Boston, MA.

Adjuvant activity is manifested by a significant increase in immune-mediated protection by development of an immune response in an individual who otherwise would not respond at all to a vaccine. Enhancement of humoral immunity is typically manifested by a significant increase in the titer of antibody raised to the antigen.

The methods of the present invention to provide administration of GM-CSF in conjunction with a vaccine has the following advantages. The total antigenic load of vaccine to be administered may be reduced since less antigen in the presence of GM-CSF would elicit an immunologic response at least equivalent to that achieved by the administration of the normal amount of the vaccine. Since less antigen would be required per vaccination by administering GM-CSF in accordance with the present invention, the probability of undesirable side-effects associated with some vaccines currently in use would be reduced.

The immune response of certain types of individuals who respond poorly to vaccination would be enhanced by administering GM-CSF in conjunction with a vaccine. Types of individual who should benefit from the methods of the present invention include (1) those types having impaired immune responsiveness, (2) those individuals who appear normal but who are nevertheless nonresponsive to certain vaccines as well as (3) individuals

undergoing immunosuppressive therapies such as radiation and chemotherapy.

Thus, we have discovered an effective method for (1) enhancing an effective primary immune response in mammals to antigens present in a vaccine, (2) enhancing an effective level of antibodies in mammals exposed to antigens in vaccines, and (3) enhancing a primary immune response in mammals to antigens present in a vaccine wherein the immune response by the mammal without the administration GM-CSF would not be strong enough or fast enough to prevent disease.

The vaccines contemplated for use in accordance with the present invention include but are not limited to bacterial vaccines, toxoid vaccines (inactivated toxins) and viral vaccines or mixtures thereof used for active immunization. See for example chapter 75 entitled "Immunizing Agents" in Remington's Pharmaceutical Sciences 14th Edition 1990 Mack Publishing Co. p 1426-1441 and the antitoxins, toxoids, vaccines and live vaccines approved by the U.S. Food and Drug Administration and listed on page 208-209 (Product Category Index) of the Physician's Desk Reference, 46th Ed. 1992. Suitable bacterial vaccines include bacterial vaccines against the following disease entities or states: cholera, pertussis, plague, typhoid fever, meningitis, pneumococcal pneumonia, *H. influenzae* type B, leprosy, gonorrhea, Group B meningococcus, and Group B streptococcus, Gram-negative sepsis, *E. coli* sepsis, and *Pseudomonas aeruginosa*. Suitable toxoids include diphtheria toxoid, botulism toxid, and tetanus toxoid. Suitable viral vaccines include live and inactivated viral vaccines against the following disease entities or states: poliomyelitis, measles rubella, yellow fever, mumps, hepatitis B, hepatitis C and viral influenza.

The suitable "multiple antigens" include diphtheria and tetanus toxoids, the triple antigen-diphtheria, pertussis and tetanus

toxoids such as are available from Connaught Laboratories, Inc. Swiftevater, PA 18370. The wide variety of viral strains and cell substrates and the the varied immunization schedules used in different countries are disclosed in White, D.O., and Fenner F., Medical Virology 3rd Edition (Academic Press 1986).

In addition, the GM-CSF will typically be used to enhance the protection afforded by animal or human vaccines that are considered "weak" (i.e., provide diminished protection in terms of level, extent, and/or duration). Examples of such vaccines are bacterins such as Bordetella bacterin, *Escherichia coli* bacterins, Haemophilus bacterins, Leptospirosis vaccines, *Moraxella bovis* bacterin, Pasteurella bacterin and *Vibrio fetus* bacterin, pneumococcal vaccines and attenuated live or killed virus products or recombinant antigenic viral products such as hepatitis B, influenza A & B, bovine respiratory disease vaccine, infectious bovine rhinotracheitis, parainfluenza-3, respiratory syncytial virus, bovine virus diarrhea vaccine, equine influenza vaccine, feline leukemia vaccine, feline respiratory disease vaccine rhinotracheitiscalicipneumonitis viruses, canine parovovirus vaccine, transmissible gastroenteritis vaccine, pseudorabies vaccine, and rabies vaccine.

The term "effective amount" as used herein regarding the effective amount of GM-CSF administered in accordance with the present invention means an amount of GM-CSF which produces an increase in antibody level sufficient to provide increased protection from an infectious agent than if a vaccine had been administered without GM-CSF. However, it should be noted a significant increase in antibody level may be relatively small. The effective amount of GM-CSF administered is from 0.1 to 500 μg of GM-CSF per kilogram of body weight. More preferably, the effective amount administered is from 1 μg to 100 μg and most preferably from 5 to 50 μg of GM-CSF per kilogram of body weight.

The amount, frequency and period of administration will vary depending upon factors such as the level of the specific antibody titers, the class of antibody to be induced, the vaccine type as well as the age of the patient and general physical condition. The GM-CSF can be administered before, concurrently with or after the vaccine is administered. Preferably, one dose of GM-CSF is given to the patient from 1 to 14 days prior to the administration of the vaccine. Most preferably the GM-CSF is administered about 24 hours prior to or after the administration of the vaccine.

The GM-CSF will normally be administered separately from the vaccine, although it may be administered in combination with the vaccine. When GM-CSF is combined with the vaccine, the composition administered contains an immunogen that is effective in eliciting a specific response to a given pathogen or antigen, a pharmaceutically acceptable vaccine carrier and an immunopotentiating amount of GM-CSF. Administration of GM-CSF can be subcutaneous, intravenous, parenteral, intramuscular, or any other acceptable method. Preferably, GM-CSF is administered prior to the administration of the vaccine and at the same site where the vaccine is to be administered. The formulations and pharmaceutical compositions contemplated by the above dosage forms can be prepared with conventional pharmaceutically acceptable excipients and additives, using conventional techniques. Other adjuvants may be administered either with the vaccine or together with the GM-CSF.

If multiple doses of the vaccine are to be administered over a period of time, additional GM-CSF may be administered in conjunction with each subsequent dose of the vaccine. The amount of GM-CSF which is administered with each subsequent dose of the vaccine may be more, the same or less than the amount of GM-CSF administered in conjunction with the initial dose of the vaccine. The amount of GM-CSF administered with each subsequent dose of the

vaccine will depend upon the antibody response of the patient after the first dose of the vaccine.

Solutions of GM-CSF to be administered may be reconstituted from lyophilized powders and they may additionally contain preservatives buffers, dispersants, etc. Preferably, GM-CSF is reconstituted with any isotonic medium normally utilized for subcutaneous injection, e.g., preservative-free sterile water.

A sustained release formulation of GM-CSF can be administered which will result in a longer serum half-life of the drug. Examples of such formulations are the following:

Formulation 1

INGREDIENTS

Lyopilized GM-CSF	10 - 1000 mcg
Zinc Acetate	4.0 mg
Protamine Sulfate	2.5 mg
Sodium Hydroxide	0.6 mg
Water for Injection q.s.	1 ml

To prepare the sustained release preparation of GM-CSF according to Formulation 1, the lyophilized GM-CSF is dissolved in a portion of the Water for Injection and the pH of the solution is adjusted to 8.2 using sodium hydroxide. The protamine sulfate is added and the mixture is agitated, after which the zinc acetate is added and the mixture is again agitated. The total solution is brought to the final volume with the remaining Water for Injection. Preferably, the sodium hydroxide, protamine sulfate and zinc acetate are added as concentrated aqueous solutions (e.g. for protamine, 100 microliters of a 25 mg/ml aqueous solution).

Formulation 2

INGREDIENTS

Lyopilized GM-CSF	10 - 1000 mcg
Water-for-injection for reconstitution	0.2 ml
Dioctyl Sodium Sulfosuccinate	1 mg
Peanut oil for emulsion	2 ml
Peanut oil for gel	2 ml
Aluminum monostearate	50 mg

To prepare the sustained release preparation of GM-CSF according to Formulation 2, the aluminum monostearate is mixed into the peanut oil for the gel and heat elevated to form the gel according to known methods.

The dioctyl sodium sulfosuccinate is dissolved into the Water for Injection. The lyophilized GM-CSF is reconstituted with the dioctyl sodium sulfosuccinate solution, the resultant solution is transfered into the peanut oil for emulsion and mixed by vortexing. The resultant emulsion is then mixed into the previously prepared gelled peanut oil and mixed by vortexing.

Formulation 3

INGREDIENTS

Lyopilized GM-CSF	10 - 1000 mcg
Copper Acetate	0.2 mg
Sodium Phosphate, Dibasic	2.27 mg
Sodium Phosphate, Monobasic	0.55 mg
Sodium Hydroxide	0.6 mg
Water for Injection q.s.	1 ml

To prepare the sustained release preparation of GM-CSF according to Formulation 3, the monobasic and dibasic sodium phosphates are dissolved in a portion of the Water for Injection. The lyophilized GM-CSF is then dissolved in this solution and the pH is adjusted to 7.8 with the sodium hydroxide. The copper acetate is then added and the solution is agitated. The solution is brought to final volume using the remaining Water for Injection. Preferably, the sodium hydroxide and copper acetate are added as concentrated aqueous solutions (e.g., for copper acetate, 100 microliters of a 2 mg/ml aqueous solution).

Additional Sustained Release Formulations

Additional sustained release formulations of GM-CSF can be prepared using micoencapsulated or microspheres of GM-CSF prepared using polymers such as polyanhydrides, polyphosphazenes, collagen, alginates, poly(methacrylates), gelatin, poly(hydroxybutyrate), poly(caprolactone), ethylene vinyl acetate or polylactide glycolide.

Sustained release GM-CSF can also be prepared as chemical conjugates of GM-CSF using polyethylene glycol, dextran poly(aminoacids) and other similar polymers.

The effect of GM-CSF on enhancing the immune response of a vaccine is illustrated by the following non-limiting human clinical data which should not be construed to limit the scope of the disclosure.

Example 1

Recombinant human GM-CSF was shown to enhance the efficacy of recombinant hepatitis B vaccine on dialysis patients who had not responded to the hepatitis B vaccine.

The objective of the present experiment was to determine whether the co-administration of GM-CSF and hepatitis vaccine would be capable of restoring immunologic responsiveness to patients

with renal failure who had been previously unresponsive to hepatitis vaccination.

Fifteen dialysis patients who had not responded to at least 3 attempts at vaccination with hepatitis B vaccine as determined by their antibody titers against the hepatitis B surface antigen (HBsAg) were treated with GM-CSF. Six patients were injected subcutaneously with 0.5 micrograms (μg) of GM-CSF per kilogram (kg) weight of the patient, (produced in an *E. coli.* expression system by Schering-Plough, Kenilworth, New Jersey, USA), five patients with 5 μg of GM-CSF per kg weight of the patient and four patients with 10 μg of GM-CSF per kg weight of the patient, and the site of injection was marked. Twenty-four hours after administration of the GM-CSF, the patients were each administered 40 μg of the hepatitis B vaccine, HBVax ® (Merck, Sharpe and Dohme, Gmbh, Darmstadt, Federal Republic of Germany) at the same site as the GM-CSF was injected. Four weeks after administration of the vaccine, blood samples were drawn from the patients and the samples were tested for the presence of anti-hepatitis B antibody. The results are shown in the table below.

TABLE 1

Dosage of GM-CSF (µg/kg wt.)	Antibody titer against HBsAg Units*/liter
0.5	0
0.5	0
0.5	0
0.5	0
0.5	0
0.5	710
5.0	0
5.0	35
5.0	125
5.0	920
5.0	2600
10.0	0
10.0	0
10.0	440
10.0	7240

* A unit is defined as the reciprocal of the serum dilution which produced a half maximal response in a standard ELISA.

As can be seen the data presented above, GM-CSF was an effective adjuvant used in conjunction with the hepatitis B vaccine.

Example 2

Recombinant human GM-CSF was shown to enhance the efficacy of viral influenza vaccine in elderly patients.

Human influenza viruses, occurring during pandemics (Influenza A) and epidemics (Influenza A and B), cause significant excess morbidity and morality in the elderly, not only in those with underlying chronic diseases, but also in apparently healthy subjects. Since influenza vaccine has been shown to provide benefits in reducing both morbidity and mortality, flu vaccine is strongly recommended in subjects at high risk to develop flu related complications, and substantial resources are expended annually in an effort to vaccinate high-risk subjects .

However, despite large immunization programs, influenza remains a significant cause of illness and death in the elderly. Several methods, such as administering two to three times the standard vaccine dose or giving a booster dose one month after a first standard dose have not been shown to improve immunoresponse to flu vaccine in the elderly.

Accordingly, a double-blind, placebo-controlled, dose escalation study was carried out to determine whether the immunoresponse to flu vaccine is enhanced by the administration of recombinant GM-CSF. Five different dosages of recombinant GM-CSF (produced in an *E. coli.* expression system by Schering-Plough, Kenilworth, New Jersey, USA) were tested, namely, 0.25, 0.5, 1, 2.5 and 5 μg/kg in comparison to placebo. Sixty elderly healthy subjects were enrolled. They received recombinant GM-CSF or a placebo subcutaneously in one arm just before the intramuscular administration of the French 1992-1993 trivalent flu vaccine (A/Singapore/6/86 [H1N1], A/Beijing/353/89 [H3N2] and B/Yamagata/16/88) in the other arm (Pasteur Vaccins, Marnes-la-Coquette, France). Specific hemoagglutinin-inhibiting (HAI) antibody titers against the three flu virus strains were determined at baseline and 1, 3 and 6 weeks after vaccination. None of the 15 patients who received placebo with the flu vaccine showed simultaneous seroconversion to all the three strains of the flu vaccine,

whereas 5 (56%) and 3(33%) of the 9 patients receiving 2.5 and 5 μg/kg of recombinant GM-CSF seroconverted to all three strains.

The protocol which was used is described in more detail below.

Materials and Methods

Subject Selection. Subjects were healthy elderly people of both sexes and of at least 65 years of age. Volunteers were screened by medical history, and laboratory tests that included a complete blood cell count, biochemistry, urinalysis and serology. Subjects had to have a baseline hemoagglutinin-inhibiting (HAI) antibody titers ≤ 1:40 for the A-H1N1 and B influenza strains and ≤ 1:80 for the A-H3N2 influenza strain contained in the 1992-1993 French flu vaccine. Subjects with history of severe or unstable chronic illness or malignancy, taking antineoplastic or immunosuppressive drugs, with significantly abnormal results of the screening laboratory tests, allergic to eggs, with history of an influenza like illness in the past six months, or acutely ill at the time of specimen collection were excluded from the study.

Study Design. The study was designed as a double-blind, placebo-controlled, dose finding study.

Drug administration and vaccination. The 60 subjects enrolled in the study were divided into five dose groups of 12 subjects each. Each group received a single dose of 0.25, 0.5, 1, 2.5 or 5 μg/kg of r GM-CSF or placebo. Recombinant GM-CSF or placebo were administered subcutaneously in the deltoid area of the right arm, immediately after which all the subjects received 0.5 ml of a licensed 1992-1993 trivalent subvirion vaccine that contained 15 μg each of HAs from A/Singapore/6/86 (H1N1), A/Beijing/353/89 (H3N2) and B/Yamagata/16/88 viruses (Pasteur Vaccins, Marnes-la-Coquette, France) intramuscularly in the deltoid of the left arm.

Specimen collection. Blood serum and throat swabs for the virological cultures were obtained from each subject before the start of the study and 1,3 and 6 weeks after vaccination.

Serum antibodies. HAI antibodies to influenza A/Singapore (NH1N1), A/Beijing (H3N2) and B/Yamagata virus antigens were measured in serum specimens by a standard hemo-agglutination inhibition assay. Results for the preliminary analysis of the sera collected until Week 6 were obtained by testing all specimens on the same day using identical reagents. The initial starting dilution was 1:20. HAI antibody titers less than 1:20 were defined as "Not detectable" titers.

Statistical analysis. Success according to the antibody titers were defined in two different ways:

- Seroconversion: a four-fold increase of the HAI antibody titers over baseline at Week 6.
- Seroprotection: HAI antibody titers greater than baseline and at least equal to 1:40 at Week 6.

The number of successes based on these definitions were analyzed using the Fisher's exact tests. The placebo groups of the different dose groups were pooled together.

Results

Subject characteristics. All the 60 healthy elderly volunteers enrolled in the study completed the assessment at the 6th week. Table 3 reports HAI antibody titers at baseline. Most of the subjects had baseline HAI antibody titers below protective levels, that is below 1:40. Distribution of the baseline titers was similar among all treatment groups, with the exception of the group treated with 0.5 µg/kg in which most of the patients had no detectable antibody titers.

Immunoresponse. Table 4 shows the number of patients who seroconverted to all three strains 6 weeks after the administration of GM-CSF/placebo and flu vaccine. Results are also reported separately for each strain.

None of the 15 subjects who received placebo with the flu vaccine showed simultaneous seroconversion to all three flu virus strains, whereas 5 of 9 (56%) and 3 of 9 (33%) of the subjects treated with 2.5 and 5 μg/kg of the rGM-CSF were seroconverted to all three strains. In addition, when the results are examined separately for each flu strain, seroconversion rates for 2.5 and 5 μg/kg of rGM-CSF are consistently higher (ranging from 44% to 67%) than those observed with placebo (ranging from 13% to 20%). Interestingly, seroconversion rates observed with the lowest dose (0.25 μg/kg) have been higher than those observed with 0.5 and 1 μg/kg.

As shown in Table 5, the conclusion of the results do not change also using "seroprotection" as definition of success. As expected, seroprotection rates are higher than those of seroconversion in all treatment groups, because seroprotection is easier to accomplish than seroconversion. Again, patients treated with rGM-CSF showed a higher immunoresponse rate to the flu vaccine than those treated with placebo. In fact, 5 of 9 (56%) and 3 of 9 (33%) patients treated with 2.5 and 5 μg/kg were protected against all three strains, whereas only 1 of the 15 subjects (7%) treated with placebo was seroprotected against all three strains.

Table 2: ***Study design.***

	Number of subjects	
	CSF 39300	Placebo
Group I: 0.25 µg/kg	9	3
Group II: 0.5 µg/kg	9	3
Group III: 1. µg/kg	9	3
Group IV: 2.5 µg/kg	9	3
Group V: 5. µg/kg	9	3

Table 3: ***HAI antibody titers at baseline.*** The table reports the number of subjects in each treatment group according to baseline HAI antibody titers for each of the three flu strains.

	Number of subjects with specific HAI antibody titers at baseline									
	A/Beijing [H3N2]				A/Singapore [H1N1]			B/Yamagata		
Baseline HAI antibody titers:	<1:20	1:20	1:40	1:80	<1:20	1:20	1:40	<1:20	1:20	1:40
Placebo + flu vaccine	5	6	4	-	10	4	1	10	2	3
CSF 39300 + flu vaccine										
0.25 µg/kg	4	3	2	-	6	1	2	5	4	-
0.5 µg/kg	1	5	3	-	7	1	1	4	1	4
1 µg/kg	2	4	3	-	4	3	2	4	2	3
2.5 µg/kg	2	4	1	2	6	2	1	4	5	-
5 µg/kg	9	-	-	-	8	1	-	8	1	-

Table 4: ***Results of seroconverstion.*** Seroconversion is defined as a four-fold increase of the HAI antibody titers over baseline. The second column of the table reports the number of subjects who seroconverted to all three strains contained in the flu vaccine. The last three columns show the number of seroconversion observed for each flu strain.

		Number of seroconversion observed for the diferent flu strains		
	Patients simultaneously seroconverted to all three strains	A/Beijing [H3N2]	A/Singapore [H1N1]	B/Yamagata
Placebo + flu vaccine	0/15 (0%)	3/15 (20%)	3/15 (20%)	2/15 (13%)
CSF 39300 + flu vaccine				
0.25 µg/kg	2/9 (22%)	4/9 (44%)	4/9 (44%)	3/9 (33%)
0.5 µg/kg	0/9 (0%)	2/9 (22%)	3/9 (33%)	1/9 (11%)
1 µg/kg	1/9 (11%)	2/9 (22%)	1/9 (11%)	4/9 (44%)
2.5 µg/kg	5/9 (56%)	6/9 (67%)	5/9 (56%)	5/9 (56%)
5 µg/kg	3/9 (33%)	4/9 (44%)	4/9 (44%)	5/9 (56%)

Table 5: ***Results of seroprotection.*** Seroprotection is defined as an increase of the HAI antibody titers over baseline and at least 1:40. The second column of the table reports the number of subjects who develop seroprotective HAI antibody titers against all three strains contained in the flu vaccine. The last three columns show the number of seroprotection observed for each flu strain.

		Number of seroprotection observed for the diferent flu strains		
	Patients simultaneously seroprotected to all three strains	A/Beijing [H3N2]	A/Singapore [H1N1]	B/Yamagata
Placebo + flu vaccine	1/15 (7%)	8/15 (53%)	5/15 (33%)	3/15 (20%)
CSF 39300 + flu vaccine				
0.25 µg/kg	4/9 (44%)	6/9 (67%)	5/9 (56%)	6/9 (67%)
0.5 µg/kg	1/9 (11%)	5/9 (56%)	3/9 (33%)	3/9 (33%)
1 µg/kg	2/9 (22%)	4/9 (44%)	3/9 (33%)	4/9 (44%)
2.5 µg/kg	5/9 (56%)	8/9 (89%)	5/9 (56%)	6/9 (67%)
5 µg/kg	3/9 (33%)	4/9 (44%)	4/9 (44%)	5/9 (56%)

WHAT IS CLAIMED IS:

1. A method for enhancing an immune response of a mammal to a vaccine comprising administering to a mammal in need of vaccination an effective amount of GM-CSF in conjunction with a vaccine.

2. The method of claim 1 wherein the vaccine is selected from a group consisting of hepatitis B vaccine and influenza vaccine.

3. The method of claim 1 wherein the GM-CSF which is administered is contained within a sustained release formulation.

4. The use of GM-CSF for enhancing an immune response of a mammal to a vaccine.

5. The use of GM-CSF for the manufacture of a medicament for enhancing an immune response to a vaccine.

6. The use of either claim 4 or 5 in which the he vaccine is selected from a group consisting of hepatitis B vaccine and influenza vaccine.

7. The use of either claim 4 or 5 wherein the GM-CSF is contained within a sustained release formulation.

8. A pharmaceutical composition comprising an effective amount of GM-CSF; and a vaccine.

9. The pharmaceutical composition of claim 8 wherein the GM-CSF is contained within a sustained release formulation.

10. A kit for enhancing an immunogenic response of a mammal to antigens in a vaccine comprising a container of a pharmaceutical composition of GM-CSF and a pharmaceutically acceptable carrier therefor; and a container of a pharmaceutical composition of a vaccine and a pharmaceutically acceptable carrier therefor.

11. The kit of claim 10 wherein the GM-CSF is contained within a sustained release formulation.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 93/06298

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K39/39 A61K39/29 A61K39/145 A61K9/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO,A,91 01146 (PRAXIS BIOLOGICS, INC.) 7 February 1991 see page 12, line 15 - line 29; claims 35,37-53	1,2,4-6, 8,10 3,7,9,11
X	MOLECULAR IMMUNOLOGY vol. 28, no. 3 , March 1991 , OXFORD, GB pages 295 - 299 J.W. SCHRADER 'PEPTIDE REGULATORY FACTORS AND OPTIMIZATION OF VACCINES.'	1,2,4-6, 8,10
Y	see page 299, left column, line 22 - line 32 see page 295, abstract	3,7,9,11

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 October 1993	07. 12. 93

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	RYCKEBOSCH, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter mal Application No
PCT/US 93/06298

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PATENT ABSTRACTS OF JAPAN vol. 12, no. 103 (C-485)(2950) 5 April 1988 & JP,A,62 230 729 (SUMITOMO PHARMACEUTICAL CO. LTD.) 9 October 1987 see abstract -----	3,7,9,11

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06298

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-4 and 6 and 7(both partially) are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/US 93/06298

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9101146	07-02-91	AU-A-	6055090	22-02-91
		CA-A-	2063271	15-01-91
		EP-A-	0482068	29-04-92
		JP-T-	4506662	19-11-92